American Journal of Hematology 58:273–277 (1998)

Longitudinal Analysis of Circulating Myeloma Cells Detected by Allele Specific mRNA In Situ Hybridization

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Individual myeloma cells in the peripheral blood of 7 patients with multiple myeloma were detected by mRNA in situ hybridization (ISH) using biotinylated antisense oligonucleotide probes to non-germline sequences of the CDR3 region of the immunoglobulin heavy chain gene. Peripheral blood samples from these patients were studied over a period of 2–3 years. The number of circulating myeloma cells varied from 0.1–23% of the mononuclear cell population. Longitudinal studies showed that the highest number of circulating myeloma cells were present during escape phase and thus the percentage of mRNA ISH+ cells correlated with the clinical state of the patient. This technique is the most accurate means of monitoring and quantitating individual myeloma cells in the peripheral blood of patients with myeloma and provides insight into the relevance of circulating myeloma cells. Am. J. Hematol. 58:273–277, 1998.

Key words: multiple myeloma; mRNA; in situ hybridization; anti sense; oligonucleotides

INTRODUCTION

Although archetypal plasma cells are seen infrequently in the peripheral blood of patients with multiple myeloma, a variety of techniques have now demonstrated that a small number of myeloma cells are present in the circulation of these patients [1–6]. Circulating myeloma cells detected by the presence of unique IgH gene rearrangements include immature plasma cells [7] and post germinal centre B cells [8,9]. Renewed interest in the presence of circulating myeloma cells has arisen following the increased use of autologous peripheral blood as a source of stem cells for transplantation and several reports have demonstrated that myeloma cells remain in autologous apheresis products [6] even after CD34 purification [10–12]. In addition, it has been demonstrated that the number of blood plasma cells not only correlates with disease activity but is also a significant prognostic factor at diagnosis [5]. Thus, it is important to develop accurate methods to quantitate the number of circulating myeloma cells.

Most methods used to quantitate the number of circulating myeloma cells have significant limitations. The quantitation of blood plasma cells by light chain specific immunofluorescent microscopy is labour intensive and cannot discriminate between polyclonal and monoclonal cells of the same light chain [5,13,14]. PCR techniques provide the most sensitive means of detecting minimal residual disease but they are difficult to quantitate accurately and analyse the overall content of cell populations rather than individual cells [1–3,15,16]. Flow cytometric methods can detect individual plasma cells but the CD38++CD45− populations analysed are not exclusively monoclonal as both kappa and lambda expressing cells are present [7]. Furthermore the CD38++CD45− population does not include all myeloma cells as CD38++CD45+ cells express cIg and are considered to be primitive plasma cells [17–19]. Methods that depend on the coexpression of other markers like CD56, CD19, and VLA-5 to identify myeloma cells have been suggested but have not received universal support [20,21].

We prepared anti-sense allele specific oligonucleotides from non-germline sequences of the CDR3 region of the rearranged immunoglobulin heavy chain gene of

Contract grant sponsor: National Health and Medical Research Council; Contract grant sponsor: Anthony Rothe Memorial Trust.

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Received for publication 4 November 1997; Accepted 8 April 1998

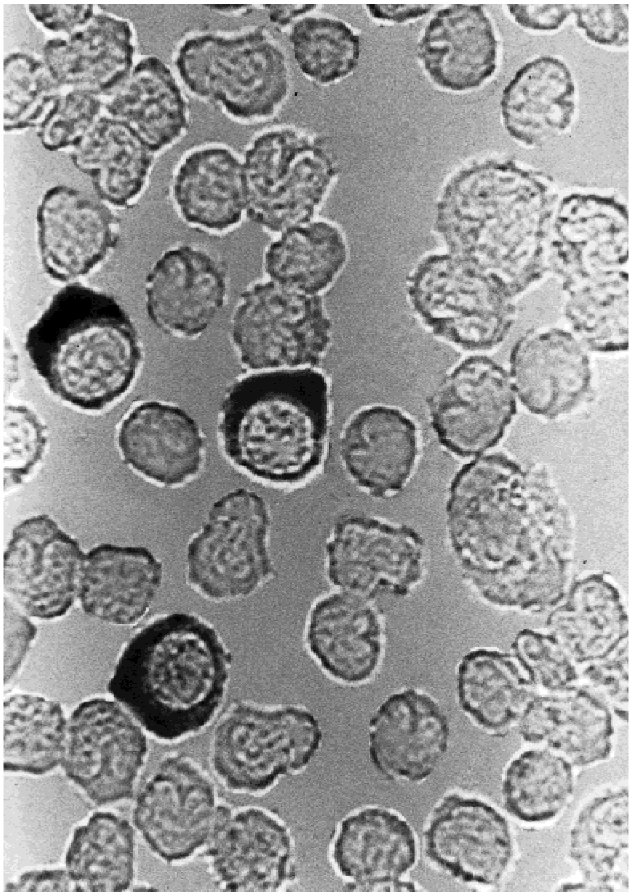

Fig. 1. Staining of peripheral blood myeloma cells in cytospin preparations with biotinylated antisense oligonucleotide sequences by mRNA in situ hybridization.

patients with myeloma [4,8]. A series of mRNA in situ hybridization (ISH) studies demonstrated the potential of these probes for identifying individual myeloma cells in the bone marrow and peripheral blood of patients with myeloma [4]. Subsequent studies that combined immunophenotyping and mRNA in situ hybridization demonstrated that only a minority of circulating B cells belonged to the malignant clone [22]. In the current study, mRNA in situ hybridization has been used to detect the presence of clonal cells in the peripheral blood of patients with myeloma over a period of 2–3 years and to determine whether changes in the number of circulating myeloma cells correspond with the stage of the disease and other established parameters.

MATERIALS AND METHODS

Patients

Peripheral blood and bone marrow samples were collected after informed consent from 7 patients during routine clinical assessment. Patients were treated according to Australian Leukaemia Study Group (ALSG) Myeloma II protocol [23], which compared intensive combination chemotherapy with or without α-interferon. A range of clinical and laboratory studies were performed at each clinic assessment to determine the stage of the disease. Mononuclear cells were separated on Ficoll-Paque, washed, and cytospin preparations made.

DNA Sequencing

Sequencing was performed as previously described [8]. Briefly, the segment of the rearranged immunoglobulin gene between J and either framework 2 or framework 3 was amplified from DNA extracted from bone marrow using consensus primers. Additional rounds of PCR were used to attach binding sites for sequencing primers USP and RSP. Monoclonal products were sequenced in both directions using an Applied Biosystems (Foster City, CA) 373A automated sequencer. Two independent amplifications were performed for each patient. Sequences were submitted to Genbank.

mRNA In Situ Hybridization

Two antisense oligonucleotide sequences (18–24 mer) containing predominantly non-germline IgH sequences were chosen for each patient. Each sequence chosen was different from any other previously published sequence (Genbank). Oligonucleotides were biotinylated during synthesis (Bresatec, Adelaide, Australia). Air-dried slides of peripheral blood cells were fixed in 3:1 ethanol and acetic acid for 30 min. Endogenous biotin was blocked with streptavidin. Hybridization of 50 μl of probe (2 μg) was performed in a hybridization buffer containing 50% formamide, 5 × SCC, 25 mg/ml Herring sperm DNA, and 10% dextran sulphate for 24 h at 42°C. The slides were washed with 2 × SCC and 0.4 × SCC at 42°C. The signal was amplified using 2 sequential applications of streptavidin and biotinylated alkaline phosphatase (DAKO ISH detection kit, Dako, Carpinteria, CA) and finally histochemically visualized using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium in N,N-dimethylformamide. Antisense probes were tested with the patient's own cells and cells from at least 3 other patients. Negative controls were run with each batch and consisted of sense probes and antisense probes from other patient's sequences [22]. When possible 1,000 cells were examined on each slide but always more than 600 cells. Antisense probes from other patients consistently failed to stain any cells.

Other Parameters

Immunoglobulin levels were determined on a Roche (Nutley, NJ) Cobas FARA, and serum thymidine kinase levels were determined by radioenzyme assay [24].

TABLE I. Non-Germline Anti-Sense Oligonucleotide Sequences Used as Probes

Patient no.	Sex/age	Isotype	Sequence of mRNA Probes
1	F68	κLC	GTT GCC ATT GTA AGA GCT G
			TAT TTG TCA GCC ACG CCG ATT CGG
2	F38	IgGλ	CAA AGT AAT ATT CTC TCG CAC
			CCG AAA TGG TAG TCT AAG
3	F82	IgGκ	GAG CGA ATA GGT TCA CAC AG
			GTA GTC AAA GTA GTC CGG TTC
4	F42	κLC	GTG GTA GTA GTG GTA ATA TTC AT
			AGG TGG ATA TAG TGG CTA CG
5	M78	IgGκ	GGG TCG TAC CAG TGA TGT AG
			GTA TAT CTC CTA ATC TCT CG
6	M65	IgAκ	ATG GTG GTG GCC CAC CCT CG
			AGG TGA ATG CCA GTC CCC GA
7	F66	IgGκ	TCA AAG TAG TAT TCG TAC CA
			GGA TCC CTT GGC CCC AGT GG

RESULTS

When the staining conditions for the mRNA in situ hybridization technique were optimised, clonal cells were clearly evident in peripheral blood cytospin preparations (Fig. 1). These mRNA ISH+ cells were present even when plasma cells were not seen by routine morphology. Negative control slides consisted of sense probes and antisense probes prepared from the rearranged sequences of other patients. Thus, the probes used were both tumour and patient specific.

Cells that were mRNA ISH+ were seen in the blood of all 7 patients who had allele specific probes prepared. Table I shows the oligonucleotide sequences used as antisense probes and the details of each patient. The incidence of mRNA ISH+ cells ranged from 0.1 to 23% of the Ficoll-Hypaque separated mononuclear cells. Longitudinal studies were performed on a total of 83 samples from these 7 patients who regularly visited the clinic over a period of more than 2 years. Three patients went from stable to progressive disease during the study period and Figures 2–4 illustrate the changes in the % mRNA ISH+ cells during the course of their disease from stable to progression (or ‘‘escape’’) compared with the M protein level, serum thymidine kinase, and chemotherapy. Serum calcium and creatinine values remained within the normal range for each patient. The time scale on Figures 2–4 does not begin at diagnosis as there was a time delay before the CDR3 sequence was determined. The other 4 patients remain in stable disease with no significant change in the number of mRNA ISH+ cells.

The patients in Figures 2 and 4 demonstrate the typical response following a course of therapy, a stable period of about 12 months and a period of progressive disease that was refractory to therapy. Bone marrow aspirates from these 2 patients demonstrated few plasma cells (<5%) in stable disease and an increased number (70–90%) prior to chemotherapy for progressive disease. The patient in Figure 3 had kappa light chain disease. He was given chemotherapy for lytic lesions and, although there was an initial response, soon entered a progressive stage characterised by new skeletal lesions and a modest increase in urinary kappa light chain excretion that decreased with multi drug therapy. For the patient in Figure 4, the onset of progressive disease was not associated with any great rise in the IgA level but rather the presence of new lytic lesions and a rising serum thymidine kinase. For each patient, the rise in mRNA ISH positive cells corresponded with clinical symptoms of progressive disease and, at least for the patients in Figures 3 and 4, preceded the rise in M-protein. During stable disease, the number of mRNA ISH positive cells was always below 2%.

DISCUSSION

The technique of mRNA in situ hybridization provides an accurate means to determine the number of myeloma cells in the peripheral blood of patients with myeloma. In this longitudinal study, the number of circulating myeloma cells varied from 0.1 to 23% of the mononuclear cells and confirmed other laboratory (serum M-protein and thymidine kinase) and clinical markers of the clinical state of the patient. The data from these studies also confirms our previous observation the serum thymidine kinase is an excellent monitor of the disease status throughout the course of the disease [24].

The number of myeloma cells in the peripheral circulation has been an issue debated for several years but there is now a consensus that plasma cell precursors exist in the circulation of virtually all patients with myeloma. There remains some controversy about the proportion of the B-cell population that belongs to the malignant clone [22,25,26], but recent studies using PCR and in situ hybridization have clearly shown that few of the CD19+ cell population belong to the myeloma clone [22,25].

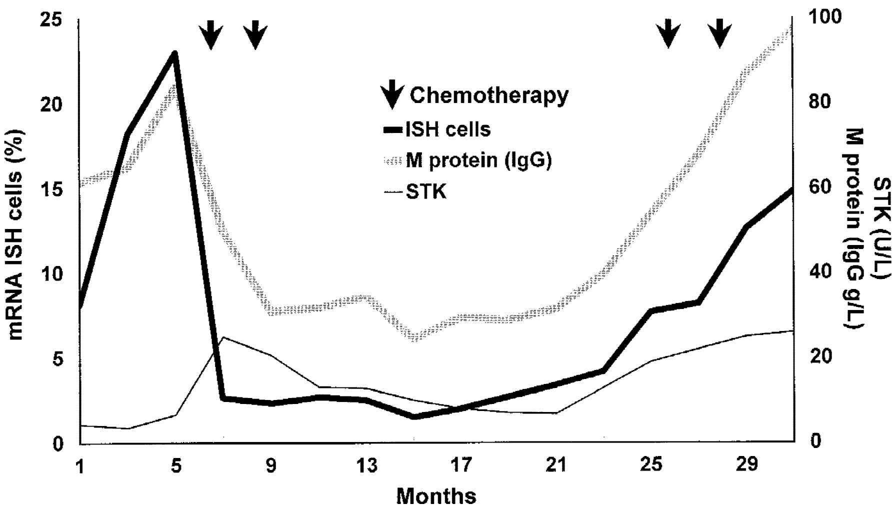

Fig. 2. Longitudinal study of mRNA ISH+ cells, serum thymidine kinase, serum M protein, and chemotherapy in a patient with IgG myeloma. The period of study includes a period of post-chemotherapy plateau and escape to progressive disease.

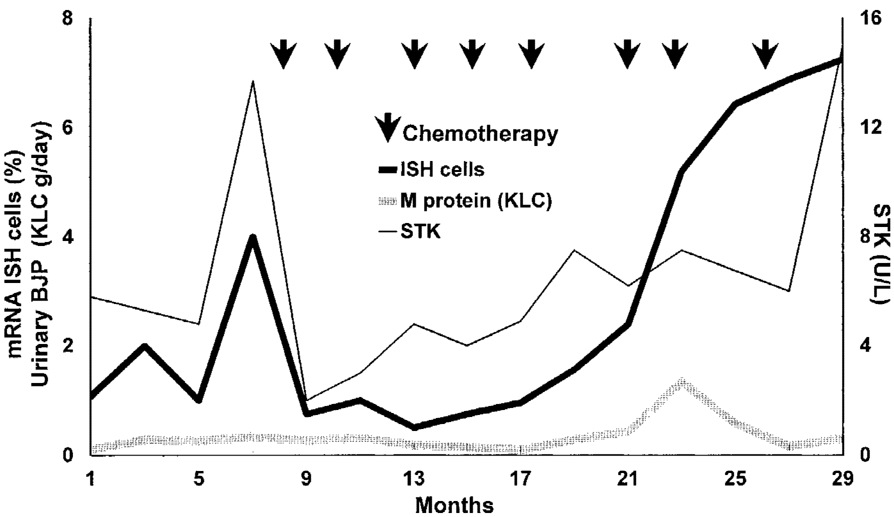

Fig. 3. Longitudinal study of mRNA ISH+ cells, serum thymidine kinase, urinary light chain, and chemotherapy in a patient with kappa light chain disease. Light chain escape correlated with a rising number of mRNA ISH+ cells in the peripheral blood.

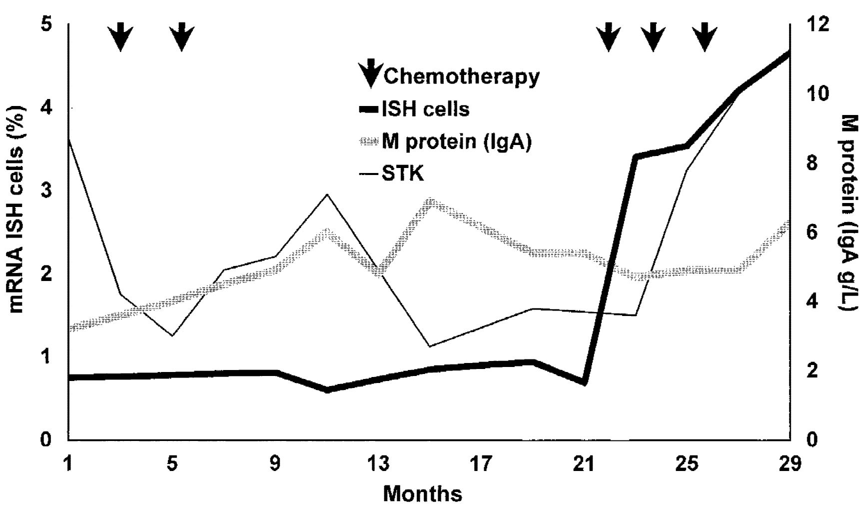

Fig. 4. Longitudinal study of mRNA ISH+ cells, serum thymidine kinase, serum M protein, and chemotherapy in a patient with IgA myeloma. Escape from plateau is clearly related to an increased number of circulating mRNA ISH+ cells.

Unlike bone marrow samples, peripheral blood CD38++ cells are predominantly polyclonal as they express both kappa and lambda light chains [7]. As immunofluorescent methods, including flow cytometry, cannot differentiate between monoclonal and polyclonal CD38++ cells of the same light chain, there must be some doubt about the accuracy of such methods to distinguish myeloma cells from polyclonal plasma cells. The antisense

probes used in this study proved to be patient and tumour specific, and unlike PCR methods, individual cells were able to be analysed. Unless the number of circulating myeloma cells is reduced below the sensitivity threshold of the ISH technique (which is about 0.1% of the leucocyte count), this method appears to be the most accurate and specific method available for monitoring individual myeloma cells in the peripheral blood. It is clear that, using routine morphology, the presence of myeloma cells in peripheral blood is underestimated. The technique could be further enhanced when used in conjunction with automated scanning microscope technology. However it is doubtful that this technique would be cost-effective in the routine clinical setting and would certainly be too complex for most routine diagnostic laboratories at this stage.

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